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## Gestational exposure to sidestream (secondhand) cigarette smoke promotes transgenerational epigenetic transmission of exacerbated allergic asthma and bronchopulmonary dysplasia §

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### Abstract

Embryonic development is highly sensitive to xenobiotic toxicity and in utero exposure to environmental toxins affects physiological responses of the progeny. In the US, the prevalence of allergic asthma is inexplicably rising and in utero exposure to cigarette smoke (CS) increases the risk of allergic asthma (AA) and bronchopulmonary dysplasia (BPD) in children and animal models. We reported that gestational exposure to sidestream (secondhand) CS (SS) promoted nicotinic acetylcholine receptor-dependent exacerbation of AA and BPD in mice. Recently, perinatal nicotine injections in rats were reported to induce peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ )-dependent transgenerational transmission of asthma. Herein, we show that F1 and F2 progeny from gestationally SS-exposed mice exhibit exacerbated AA and BPD that is not dependent on the decrease in PPAR $\gamma$  levels. Lungs from these mice show strong eosinophilic infiltration, excessive Th2 polarization, marked airway hyperresponsiveness, alveolar simplification, decreased lung compliance, and decreased lung angiogenesis. At the molecular level, these changes are associated with increased RUNX3 expression, alveolar cell apoptosis, and the antiangiogenic factor GAX, and decreased expression of HIF-1 $\alpha$  and pro-angiogenic factors NF- $\kappa$ B and VEGFR2 in the 7-day F1 and F2 lungs. Moreover, the lungs from these mice exhibit lower levels of micro-RNA (miR)-130a and increased levels of miR-16 and miR-221. These miRs regulate HIF-1 $\alpha$ -regulated apoptotic, angiogenic, and immune pathways. Thus the

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intergenerational effects of gestational SS involve epigenetic regulation of HIF-1 $\alpha$  through specific miRs contributing to increased incidence of AA and BPD in the progenies.

### Keywords

Allergic asthma; Bronchopulmonary dysplasia; Secondhand cigarette smoke; Epigenetics; micro-RNAs

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## INTRODUCTION

Epidemiological studies suggest that some environmental toxicants affect fetal development and increase the susceptibility to lung diseases (1). Furthermore, exposure to some xenobiotics during pregnancy may adversely affect the health of children; some of these effects are transmittable to subsequent generations (2, 3). Maternal smoking during pregnancy remains relatively common (4, 5) and the risk of cigarette smoke (CS)-associated pulmonary complications are highest in the lungs that are exposed to CS in utero (6–9). Epidemiological evidence and animal studies suggest that exposure to mainstream CS or environmental/sidestream CS (SS) during pregnancy adversely affects the pulmonary health of the offspring, including a higher risk for allergic asthma and airway remodeling (7, 10–14) and bronchopulmonary dysplasia (BPD) (15–17). Moreover, CS exposure either during first (18) or third trimester (19) increases the risk of wheeze and asthma in children. We have reported that gestational exposure to SS promotes BPD-like alveolar simplification and marked exacerbation of allergen-induced Th2 polarization and severity of allergic asthma (AA) in mice (20, 21). Normal fetal development occurs under hypoxic conditions and HIF-1 $\alpha$  is important for normal organogenesis (22, 23); however, HIF-1 $\alpha$  expression is significantly reduced in gestationally SS-exposed animals (17). HIF-1 $\alpha$  promotes angiogenesis and suppresses apoptosis; however, in utero SS-exposed mouse lungs have impaired angiogenesis and alveolarization and increased number of apoptotic epithelial cells in the airways (17, 21).

The prevalence of asthma in the US has been rising steadily, particularly among 0–4 yr-olds, where the incidence nearly tripled between 1980 and 1994 (24). Maternal smoking during pregnancy causes epigenetic changes, including methylation of some asthma-related genes (25). Increasing evidence suggests that lung diseases induced through in utero exposure to environmental toxins, are potentially transmitted transgenerationally (2, 3). Epidemiological evidence suggests that women smoking during pregnancy not only increase the risk of asthma in their unborn children but also among grandchildren (26). The mechanisms by which the transgenerational effects of smoking on asthma are manifested are not clear, but epigenetic mechanisms might be implicated (27). Moreover, perinatal exposure of rats to nicotine promotes development of asthma transgenerationally and the response is associated with decreased PPAR $\gamma$  through methylation of its promoter (28–30). In this communication, we show that gestational SS exacerbates AA and BPD transgenerationally through epigenetic mechanisms; however, the effects are independent of PPAR $\gamma$  but linked to specific microRNAs (miRs) that regulate apoptosis and angiogenesis.

## MATERIALS AND METHODS

### Animals

Pathogen-free BALB/c mice (FCR Facility, Frederick, MD) were kept in exposure chambers and maintained at  $26 \pm 2^\circ\text{C}$  and 12-hour light/dark cycle. Food and water were provided *ad libitum*. All animal protocols were approved by the Institutional Animal Care and Use Committee.

### Reagents

Buffers and Precast gels for the Western blot analysis were obtained from Bio Rad Laboratories Inc. (Hercules, CA). Specifics for other reagents including antibodies are provided in the description of methods

### Gestational exposure to sidestream cigarette smoke (SS)

Adult (3–4 month old) male and female mice were separately acclimatized to SS or filtered air (FA) for 2 weeks, and then paired for mating under the same exposure conditions. Mice were whole-body exposed to SS or FA for 6 hours/day, 7 days/week using Type 1300 smoking machine (AMESA Electronics, Geneva, Switzerland) as described previously (Singh et al., 2013). The machine generated two  $70 \text{ cm}^3$  puffs/min from 2R1 cigarettes (Tobacco Health Research Institute, Lexington, KY). The dose of SS was approximately equivalent to the amount of SS a pregnant woman would receive by sitting in a smoking bar for 3 hr/day throughout the gestational period (21). After pregnancy was established, male mice were removed and the pregnant mice continued to receive SS or FA until the pups were born. Mice were sacrificed by an intraperitoneal injection of 0.2 ml Euthasol.

### Sensitization with *Aspergillus fumigatus* proteins (Af)

Lyophilized culture filtrate preparation of *Aspergillus fumigatus* (Greer, Lenoir, NC) was used to elicit allergic response in the animals. Mice (7 weeks old) were immunized intratracheally with 0.1 ml Af (50  $\mu\text{g}$ ) in endotoxin-free sterile saline or sterile saline alone) and subsequently challenged intratracheally with 0.1 ml of Af (250  $\mu\text{g}$ ) three times at 5-day intervals (Singh et al., 2009). Control animals were given sterile saline.

### Measurement of airway resistance and lung compliance

At 48 h after the last Af/saline administration, airway resistance ( $R_L$ ) and dynamic compliance ( $C_{\text{dyn}}$ ) were measured by the FlexiVent system (SCIREQ, Montreal, Quebec, Canada). Briefly, mice were anesthetized by i.p. injection of avertin (250 mg/kg). A small incision was made in the trachea through which a 20-gauge needle hub was inserted and saline-filled catheter was placed in the esophagus via the mouth to obtain transthoracic pressure. The mouse was placed on the FlexiVent apparatus and ventilated through the tracheal cannula. Doxacurium (0.5 mg/kg) was administered i.p., and heart rate and electrocardiogram were monitored by a Grass Instruments Recorder with Tachograph.  $R_L$  was assessed using increasing doses of aerosolized methacholine (MCh).  $R_L$  and  $C_{\text{dyn}}$  were derived by manipulation of ventilatory patterns and measurement of upstream and

downstream pressures. The peak responses at each MCh concentration were used for data analysis.

### **Bronchoalveolar lavage fluid (BALF) collection, cell differential, and cytokine analysis**

Established protocols were followed to obtain BALF. Briefly, mice were anesthetized and killed by exsanguination at 48 h post last Af challenge. Before excision of the lungs, the trachea was surgically exposed, cannulated and, while the left lung lobe was tied off with a silk thread, the right lobe was lavaged twice with 1 ml sterile Ca<sup>2+</sup>/Mg<sup>2+</sup> free PBS (pH 7.4). Aliquots were pooled from individual animals. Cell differentials and cytokines assays were performed as described previously (13, 20). Number of macrophages, neutrophils, lymphocytes, and eosinophils (Eos) was determined microscopically by counting at least 300 cells/sample. BALF was analyzed for IL-13 and IFN- $\gamma$  using the Mouse Cytokine ELISA kit (Biosource-Invitrogen, Camarillo, CA) according to the manufacturer's directions. The sensitivity of the assay was <10 pg/ml.

### **Preparation of lung tissues**

Mice were sacrificed and the left lung was inflated and kept in 10% formalin bath at 20 cm pressure for 24 h (21). After washing, the lungs were embedded in paraffin and 5  $\mu$ m thick sections were cut for histopathology and immunohistochemistry. The right lung was quickly frozen in liquid-N<sub>2</sub>, and kept at -80°C for making lung homogenates and RNA.

### **Immunostaining**

H&E staining was performed on deparaffinized tissue sections using standard protocols (21). Computer-selected random areas of H&E-stained lung sections were used to quantitate alveolar volumes using NanoZoomer Digital Pathology slide scanner (Hamamatsu K. K. Photonics, Hamamatsu City, Japan). The analysis was done blind.

### **Immunofluorescence staining for HIF-1 $\alpha$ , NF- $\kappa$ B, and CD31**

After deparaffinization, immunofluorescence (IF) staining was performed as described previously (17, 21). Lung sections were stained with anti-HIF-1 $\alpha$  antibody (Cat#: ab16066, Abcam) or anti-phospho-p65-NF- $\kappa$ B (pRelA) antibody (Cat#: 3033, Cell Signaling Tech). The slides were counter stained with anti-rabbit Alexa 564-conjugated secondary antibody (Cat#: A-11010, Life Technologies). Staining for CD31 (endothelial cell marker) was performed using rabbit anti-CD31 antibody (Cat#: ab28364; Abcam) at 1:2,000 dilution followed by DyLight 549-conjugated anti-rabbit antibody (Singh et al., 2013). HIF-1 $\alpha$ -, p65-, and CD31-positive cells in the lungs were counted blind using Axioplan 2 imaging (Hamamatsu, Japan) on computer selected 9000  $\mu$ m<sup>2</sup> areas. Nuclei were stained with DAPI (blue fluorescence). The procedure was repeated three times with different animals.

### **TUNEL Assay**

To detect apoptotic cells, deparaffinized lung sections were stained using fluorescein In Situ Cell Death Detection kit (Cat#: 11 684 795 910; Roche Applied Science, Germany) as per manufacturer's directions. TUNEL-positive cells were counted blinded.

### Western blot analysis

Western blot (WB) analysis of lung homogenates was carried out as described previously (18, 21). Briefly, tissue samples were homogenized in RIPA buffer and the protein content of the extracts was determined by the BCA Protein Assay Kit (Pierce, Rockford, IL). The homogenates were run on SDS-PAGE on 10% precast polyacrylamide gels (Bio Rad Lab, Hercules CA). The gels were transferred electrophoretically to nitrocellulose membranes (Bio Rad Lab). The blots were incubated with anti-PPAR $\gamma$  antibody (Cat#: sc-7273, Santa Cruz Biotech). The mouse anti-actin antibody (Santa Cruz Biotech) was used as a control for a house-keeping protein. After incubating with secondary antibody, immuno-detection was performed using Amersham ECL Western Blotting Detection Reagent (GE Healthcare Bio-Science Corp. Piscataway, NJ) and the images were captured by Fujiform LAS-4000 luminescent image analyzer (FUJIFILM Corporation, Tokyo). Densitometry was used to quantitate the expression of specific proteins and the ratio of PPAR $\gamma$  to actin.

### Methylation analysis of PPAR $\gamma$ promoter

Methylation of the CpG island flanking the start site of PPAR $\gamma$  promoter was performed by direct pyrosequencing after bisulfite modification and PCR amplification (EpigenDx, Hopkinton, MA). Assays were pre-validated to ensure that there was no preferential amplification for either methylated or unmethylated DNA.

### RT-qPCR for microRNA (miRs), GATA-3 and T-bet

Total RNA isolated from F1 and F2 lungs was used to quantitate miR-16, miR-221, and miR-130a using RNeasy Plus Kit (Qiagen). Stem-loop qPCR was used with specific primers and reagents and the PCR products were amplified using TaqMan MicroRNA Assay together with the TaqMan Universal PCR master Mix and AB StepOnePlus Real Time PCR System (Applied Biosystems). The lung expression of GATA-3, T-bet and GAPDH were determined using the One-Step RT-PCR Master Mix and specific labeled primers/probes set (Applied Biosystems, Foster City, CA). The relative expression of each mRNA was calculated as previously described (13).

### Statistical analysis

Data were analyzed using Graph Pad Prism software 5.03 (Graphpad Software Inc., San Diego, CA). The student's t test was used for comparison between two groups at 95% confidence interval. Results were expressed as the means ( $\pm$  SD). A p value of  $\leq 0.05$  was considered statistically significant.

## RESULTS

### Gestational SS alters airway resistance and lung compliance in F1 and F2 mice

Gestational SS exacerbates Af-induced AA through increase in airway hyperresponsiveness (13, 20). In current experiments, dams were exposed to fresh air (FA) or SS two weeks prior to pregnancy and throughout the gestational period. Progeny derived from these mice (F1) and from mating of F1 (F2) were tested by FlexiVent system for airway resistance ( $R_L$ ) and lung compliance ( $C_{dyn}$ ) after Af sensitization. We tested a total of 8 FA- and 17 SS-exposed

mice from the F2 progeny of both sexes and compared their response to 6 FA- and SS-exposed mice from F1 generation. The magnitude of increase in airway resistance (Fig. 1A) and decrease in lung compliance (Fig. 1B) was similar in F1 and F2 animals from the SS-exposed dams. Thus, gestational SS exposure not only causes marked increase in airway hyperreactivity in F1 and F2, it also decreases lung compliance in both generations. Moreover, this transmission is independent of gender and exhibited by all the members of the F2 progeny. These results suggest that the inheritance of gestationally SS-induced asthma phenotype involves epigenetic rather than Mendelian genetic transmission.

### **Gestational SS increases allergen-induced Th2 and decreases Th1 markers in F1 and F2 lungs**

Gestational SS promotes Af-induced Th2 responses but decreases Th1 markers in F1 lungs (13, 20). To ascertain whether this propensity is transmitted to F2 progeny, following Af sensitization, BAL of F1 and F2 mice derived from FA- and SS-treated dams were analyzed for cellular composition and cytokine expression. Compared to FA, BAL from gestationally SS exposed mice had slightly but significantly higher number of total leukocytes in F1 (FA =  $7.4 \pm 0.6 \times 10^5$ ; SS =  $11.3 \pm 1.5 \times 10^5$ ,  $p=0.001$ ) and F2 (FA =  $6.3 \pm 1.4 \times 10^5$ ; SS =  $8.7 \pm 1.1 \times 10^5$ ,  $p=0.05$ ); however, compared to FA, SS-exposed F1 and F2 mice exhibited a marked difference in the cellular composition of the BAL, including the percentage of eosinophils in F1 (FA:  $7.0 \pm 3.2$ ; SS:  $63.5 \pm 10.8$ ,  $p=0.001$ ) and F2 (FA:  $8.6 \pm 3.2$ ; SS:  $67.2 \pm 2.3$ ,  $p=0.0001$ ) and macrophages: F1 (FA:  $85.4 \pm 5.7$ ; SS:  $26.5 \pm 8.9$ ,  $p=0.001$ ) and F2 (FA:  $81.3 \pm 7.1$ ; SS:  $13.2 \pm 3.8$ ,  $p=0.0001$ ). It is likely that decreased macrophages percentage in SS-exposed BAL reflects a large increase in the eosinophil count. Additionally, we measured the BAL levels of the Th2 cytokine IL-13 and the Th1 cytokine IFN- $\gamma$  by ELISA (Fig. 2A) and the lung expression of the Th2 transcription factor GATA3 and the Th1 transcription factor T-bet by qPCR (Fig. 2B). Together these results indicate that gestational SS promotes Th2 and suppresses Th1 responses in the lungs of F1 and F2 progenies.

### **Gestational SS causes alveolar simplification and impairs angiogenesis in F1 and F2 lungs**

Angiogenesis is critical for normal lung alveolarization (31) and we have shown that gestational SS impairs alveolarization and angiogenesis in the neonatal lung (17). We carried out similar experiments in F2 animals and compared the results with control (FA) and SS-exposed F1 lungs. H&E staining for alveolar architecture (Fig. 3A) and alveolar volumes (Fig. 3B) of 10 weeks old lungs showed that, compared to FA, gestational SS significantly increases the alveolar volumes in F1 and F2 lungs. Moreover, as detected by immunohistochemistry for CD31 (Fig. 4A, left panel) and its quantitation (Fig. 4A, middle panel) and qPCR analysis of VEGFR2 (Fig. 4A, right panel), gestational SS impairs angiogenesis in both F1 and F2 lungs. Normal expression of NF- $\kappa$ B is critical for angiogenesis of the developing lung (Patel et al., 2005) and, as seen by IHC staining (Fig. 4B, left panel) and its quantitation (Fig. 4B, right panel), nuclear expression of p65-NF- $\kappa$ B is strongly downregulated in the SS-exposed F1 and F2 lungs. In addition, as detected by TUNEL staining (Fig. 4C, left panel) and its quantitation (Fig. 4C, middle panel), gestational SS increases the number of apoptotic cells within the alveoli of F1 and F2 progenies. The transcription factor RUNX3 promotes apoptosis (32) and, as measured by



qPCR analysis, its expression is markedly upregulated in gestationally SS-exposed F1 and F2 lungs (Fig. 4C right panel). These data suggest that the effects of gestational SS on lung angiogenesis, alveolar development, and apoptosis are transmitted from F1 to F2 generation.

### **Gestational SS decreases HIF-1 $\alpha$ but not PPAR $\gamma$ expression in F1 and F2 progenies**

It has recently been demonstrated in rats that daily perinatal injection of nicotine aggravates asthmatic responses transgenerationally and the effects are associated with decreased lung expression of PPAR $\gamma$  through increased PPAR $\gamma$  promoter methylation (29, 30, 33). To ascertain whether gestational SS exposure affected PPAR $\gamma$  in F1 and/or F2 lungs, we measured the mRNA and protein levels as well as promoter methylation of PPAR $\gamma$  in gestationally FA- and SS-exposed mouse lungs. Surprisingly, qPCR and Western blot analysis indicated that gestational SS did not significantly alter either the mRNA or protein levels of PPAR $\gamma$  in F1 or F2 lungs (Fig. 5A left and middle panel). Similarly, pyrosequencing analysis did not show significant changes in the methylation of PPAR $\gamma$  promoter (Fig. 5B). On the other hand, there was non-significant trend for higher levels of PPAR $\gamma$  mRNA and protein in gestationally SS-exposed F1 and F2 lungs. These results suggest that the asthma phenotype induced by gestational SS-exposures in F1 and F2 lungs is not associated with epigenetic changes in PPAR $\gamma$  levels.

HIF-1 $\alpha$  controls angiogenesis as well as apoptosis and is significantly decreased in gestationally SS-exposed animals (17). We quantitated HIF-1 $\alpha$  levels in the airways of F1 and F2 animals by immunohistochemistry. Results show that HIF-1 $\alpha$  levels are significantly decreased in the airways of gestationally SS-exposed F1 and F2 animals (Fig. 5C left and right panel). Thus, decreased HIF-1 $\alpha$  levels are associated with asthma phenotype in F1 and F2 animals from gestationally SS-exposed mice.

### **Gestational SS affects miRs in F1 and F2 lungs that regulate angiogenesis and apoptosis**

Gestational SS affects apoptosis and angiogenesis in the mouse lung (17, 21) and increasing evidence suggests that miRs regulate these processes. We ascertained the effects of gestational SS on some critical miRs that are known to regulate angiogenesis and apoptosis. miR 16 and miR 221 are known to increase apoptosis and autophagy and decrease proliferation and angiogenesis in various cell types (34–36). These miRs are increased in gestationally SS-exposed F1 and F2 mice (Fig. 5D left and middle panel). Conversely, the expression of miR-130a that targets the proapoptotic factor RUNX3 and inhibits autophagy, and stimulates angiogenesis and cell proliferation (37, 38), is significantly decreased in F1 and F2 lungs from gestationally SS-exposed mice (Fig 5D, right panel). These results indicate that gestational SS affects the expression of pro- and anti-apoptotic/angiogenic miRs and these effects are transmitted to the next generation.

## **DISCUSSION**

In recent decades, AA among children has been inexplicably increasing and asthma burden in the US exceeds \$56 billion/year (39). In utero exposure to CS, prone children to allergic asthma and BPD (16, 18, 19). The asthma inducing effects of in utero CS exposure are associated with smoking by either parent, suggesting that exposure to environmental/SS

during pregnancy has the potential to affect AA in children. Moreover the grandmothers who smoke, elevate the risk of asthma in their nonsmoking granddaughters (26), highlighting the potential of transgenerational transmission of asthmatic phenotype induced by gestational SS exposure. Similarly, in a rat model of allergic asthma, Dr. Rehan's laboratory clearly demonstrated that the pro-asthmatic effects of perinatal nicotine injections were transmitted to F2 and F3 progenies and the effects were dependent on PPAR $\gamma$  and moderated by PPAR $\gamma$  agonists (29, 33). We have previously shown that gestational exposure of mice to SS induces AA and BPD-like phenotypes in mice and the effects are dependent on nicotinic acetylcholine receptors and blocked by the allosteric antagonist of nAChRs mecamylamine (17, 21). Following gestational SS, the proasthmatic and lung architectural changes in F1 mice are associated with Th2 polarization, increased apoptosis and decreased angiogenesis in the neonatal lung. In addition, gestational SS-induces changes in F1 lungs that are strongly correlated with desensitization of nAChRs and decreased levels of HIF-1 $\alpha$  (17).

Results presented herein clearly suggest that the effects of gestational SS on AA and BPD are transmitted to F2 generation; this includes a striking increase in methacholine-induced airway hyperresponsiveness and a strong decrease in lung compliance in F1 and F2 progenies. Asthma is mainly a Th2 inflammatory lung disease (40) and Th2 polarization is a hallmark of AA and asthma exacerbations (41). In mice, in utero exposure to CS causes strong Th2 polarization associated with significant increases in Th2 cytokines/Th2 transcription factors and significant decreases in Th1 cytokines/Th1 transcription factors (20). To understand the mechanism by which gestational SS regulates transgenerational transmission of asthma phenotype, we analyzed allergen-induced changes in Th1 and Th2 cytokines/transcription factors in F1 and F2 progenies derived from control and gestationally SS-exposed dams. Results indicate that SS exposure is associated with higher expression of Th2 cytokines/transcription factors such as IL-4, IL-13, and GATA3 and lower expression of Th1 cytokines/transcription factors (e.g., IFN- $\gamma$ , T-bet). In addition, our preliminary unpublished results show that gestational SS causes a significant decrease in the lung TNF- $\alpha$  and increase in IL-10 in F1 and F2; IL-10 suppress Th1 responses through inhibition of TNF- $\alpha$  and IFN- $\gamma$  (42). In addition, gestational SS-induced BPD-like changes in F1 to F2 lungs are associated with decreased angiogenesis (lower expression of CD31, VEGF, and VEGFR2). Hypoxic conditions and HIF-1 $\alpha$  are important for angiogenesis and normal fetal development (43); gestational SS downregulates HIF-1 $\alpha$  and increases airway/alveolar epithelial cell apoptosis in both F1 and F2 lungs.

Because 100% of F2 animals tested (n = 17), derived from gestationally SS-exposed F1 progeny, exhibited exacerbated asthma and BPD-like phenotype, ruled out the Mendelian mode of inheritance, suggesting that the transmission requires epigenetic mechanisms. Maternal smoking has been shown to cause DNA methylation and differential expression of thousands of genes some of which are related to asthma (25). Moreover, proasthmatic effects of perinatal nicotine (1 mg/kg) were shown to be passed on to F2 and F3 generations and linked to decreased PPAR $\gamma$  activity through PPAR $\gamma$  gene methylation in alveolar fibroblasts (28, 30). Therefore, we determined whether gestational SS altered the expression of PPAR $\gamma$  mRNA and protein, or affected its phosphorylation or methylation. Surprisingly, none of these PPAR $\gamma$  parameters were significantly decreased in the lungs of F1 or F2 animals derived from gestationally SS-exposed dams. Moreover, although, PPAR $\gamma$  agonist

rosiglitazone was shown to moderate the proasthmatic effects of perinatal nicotine administration in rats (33,). PPAR $\gamma$  ligands such as rosiglitazone, pioglitazone, and troglitazone are known to inhibit VEGF-induced angiogenesis (44, 45), but lung VEGF and lung angiogenesis are significantly decreased in the gestationally SS-exposed animals (17 and data presented herein). On the other hand, there is a trend for higher expression of PPAR $\gamma$  in F1 and F2 lungs from gestationally SS-derived animals and, as discussed later, miR-130a that targets PPAR $\gamma$  (46) is decreased in these lungs. Thus, it is highly unlikely that the proasthmatic transgenerational effects of gestational SS are regulated by PPAR $\gamma$  activity. Therefore, it is conceivable that, while both perinatal nicotine injections (29) and gestational SS inhalation (21) promote asthma in F1 and F2 progenies, the two treatments may employ distinct mechanisms.

One of the major epigenetic mechanisms for regulation of gene expression involves changes in levels of miRs (47); these non-coding RNAs bind and degrade specific mRNAs (48). Nicotine normally promotes cell proliferation through inhibition of apoptosis and stimulation of angiogenesis through upregulation of HIF-1 $\alpha$  (49); however, gestational SS downregulates HIF-1 $\alpha$  in the lung through desensitization of nAChRs (17, 21). Because gestational SS-induced changes in HIF-1 $\alpha$  are intimately correlated with asthma and BPD in both F1 and F2, we analyzed the expression of some miRs that are known to control angiogenesis and apoptosis and affect HIF-1 $\alpha$  levels in the lung (Fig. 6). Of the miRs tested, we observed significant changes in the expression of 3 miRs in both SS-derived F1 and F2 lungs. The expression of miR-16 and miR-221 was significantly elevated and these miRs target and decrease proapoptotic factors such as BIM-BAX, BAK (50–52); BIM, BIK, BAX, and BAK are increased in gestationally SS-exposed lungs (17). ZEB2 promotes epithelial-mesenchymal-transition (EMT) through downregulation of p53 and upregulation of Akt (53, 54) and inhibits the anti-angiogenic factor GAX (52). Although, as yet, we do not have direct evidence to show that gestational SS affects ZEB2; however, GAX and p53 are strongly upregulated and Akt is downregulated in SS-derived F1/F2 lungs (17 and data presented herein). Moreover, our preliminary results suggest that EMT is suppressed in SS-exposed lungs (Singh et al., unpublished results). Gestational SS decreases miR-130a that targets the anti-angiogenic factor GAX and the proapoptotic factor RUNX3 (37, Lee et al., 2015); both GAX and RUNX3 are increased in F1 and F2 lungs from gestationally SS-exposed mice. Furthermore, RUNX transcription factors have been implicated in predicting asthma associated with maternal smoking (55). miR-130a also promotes VEGF synthesis indirectly by targeting VEGF inhibitor Hoxa5. Although as yet we don't know whether Hoxa5 is upregulated in gestationally SS-exposed lungs, levels of VEGF are markedly reduced in both F1 and F2 lungs. It should be emphasized that we only examined the miRNAs that have been linked to HIF-1 $\alpha$ ; however, it is likely that gestational SS affects other miRNAs, whose potential effects on AA and BPD remain to be ascertained.

Nicotine/CS normally downregulates Th2 responses (e.g., allergic asthma, ulcerative colitis) and exacerbates Th1 diseases (56, 57); however, desensitization of nAChRs during gestational nicotine/SS exposure might reverse this pattern (17). In keeping with this prediction, F1 and F2 lungs from gestationally SS-exposed animals show higher Th2 and lower Th1 responses. Together our results suggest that the effects of gestational SS on AA and BPD are transmitted from F1 to F2 generations through epigenetic mechanisms, and

changes in specific miRs might contribute to this response by regulating the nAChR-HIF-1 $\alpha$  pathway. These possibilities are presented as a tentative schematic pathway in Fig. 6. Participation of other epigenetic mechanisms in regulating AA and BPD responses to gestational nicotine, such as DNA methylation and histone modifications can't be ruled out at this time.

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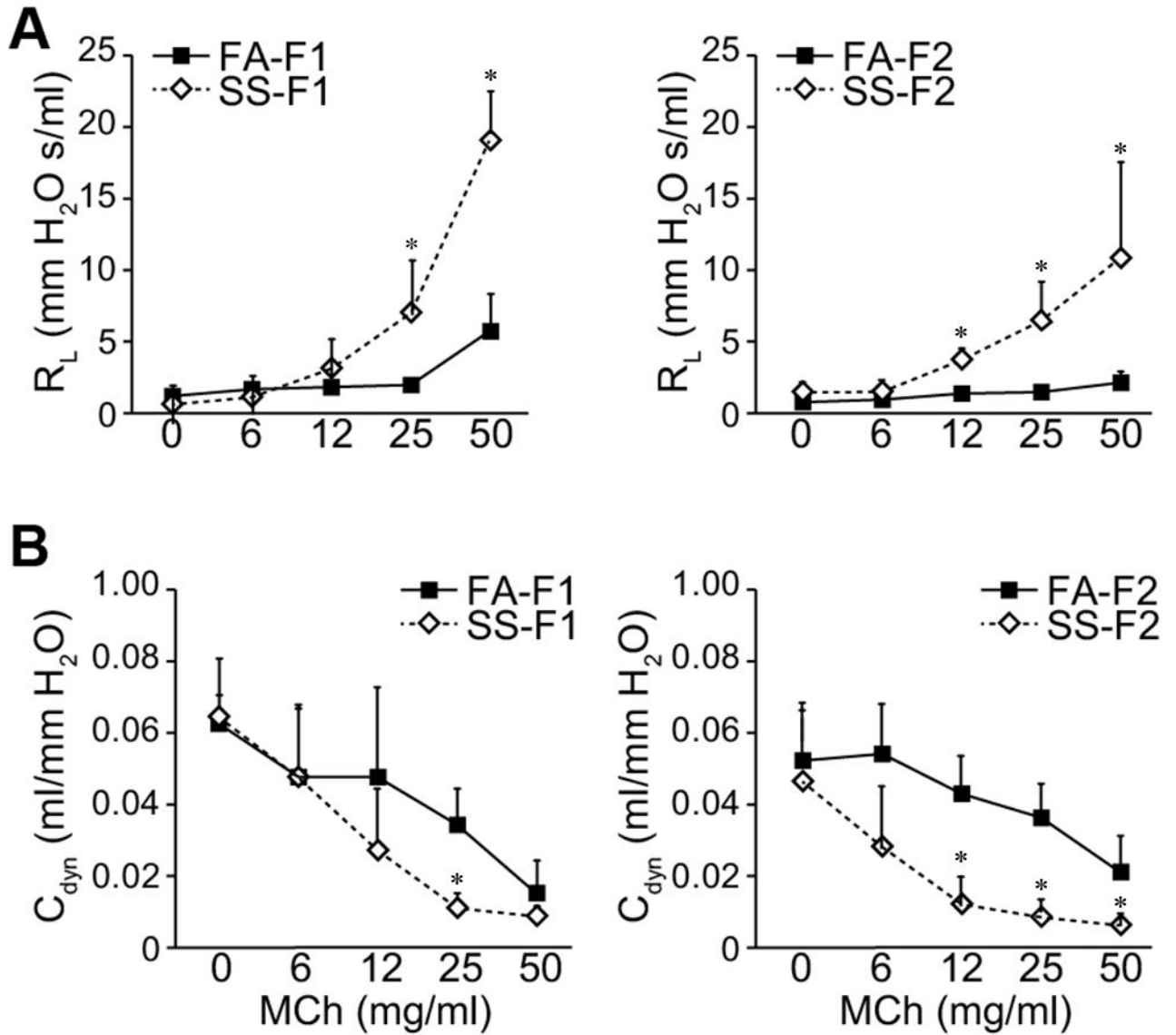
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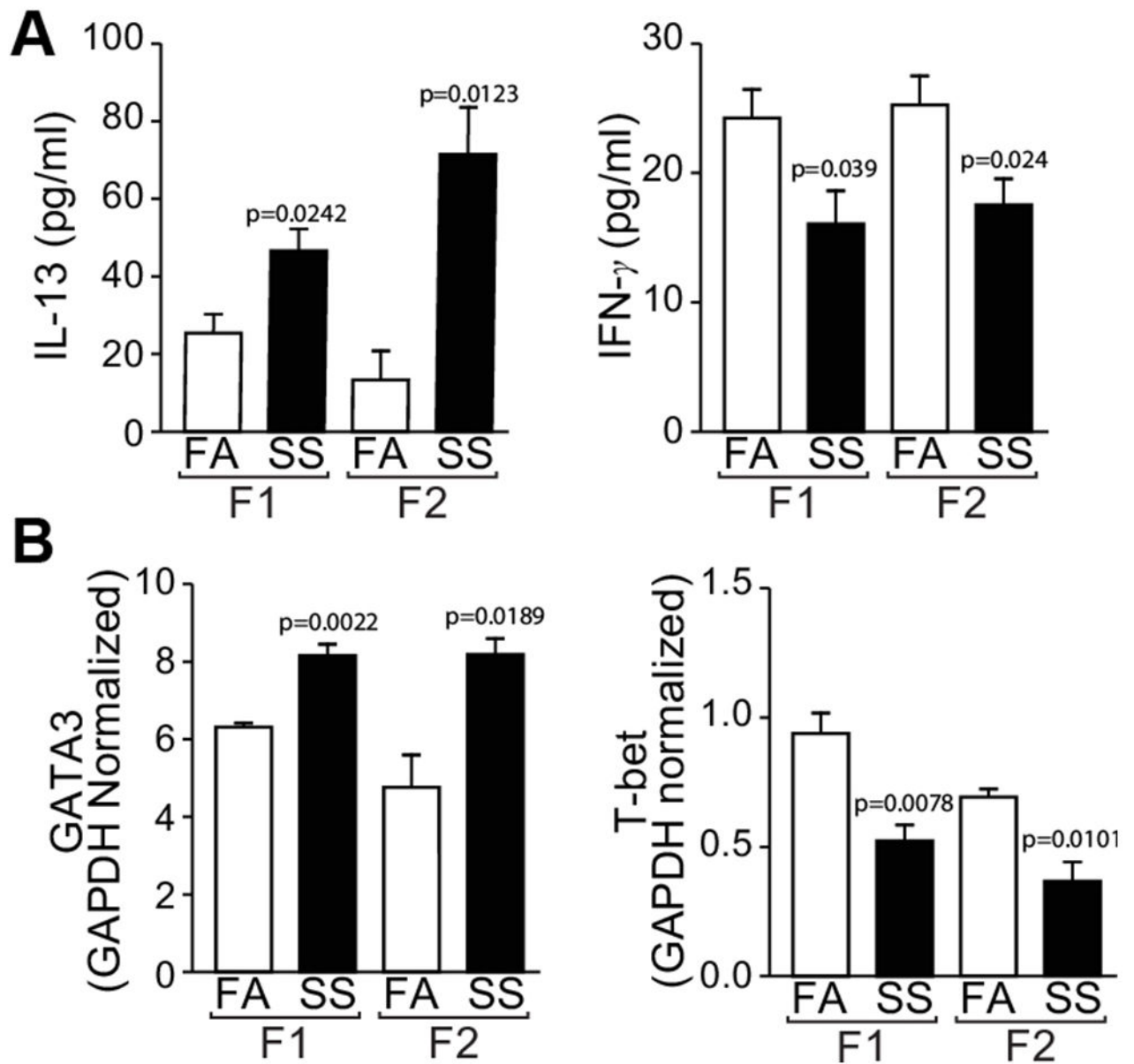
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**Fig. 1. Transgenerational effects of gestational SS on airway hyperreactivity and dynamic lung compliance**

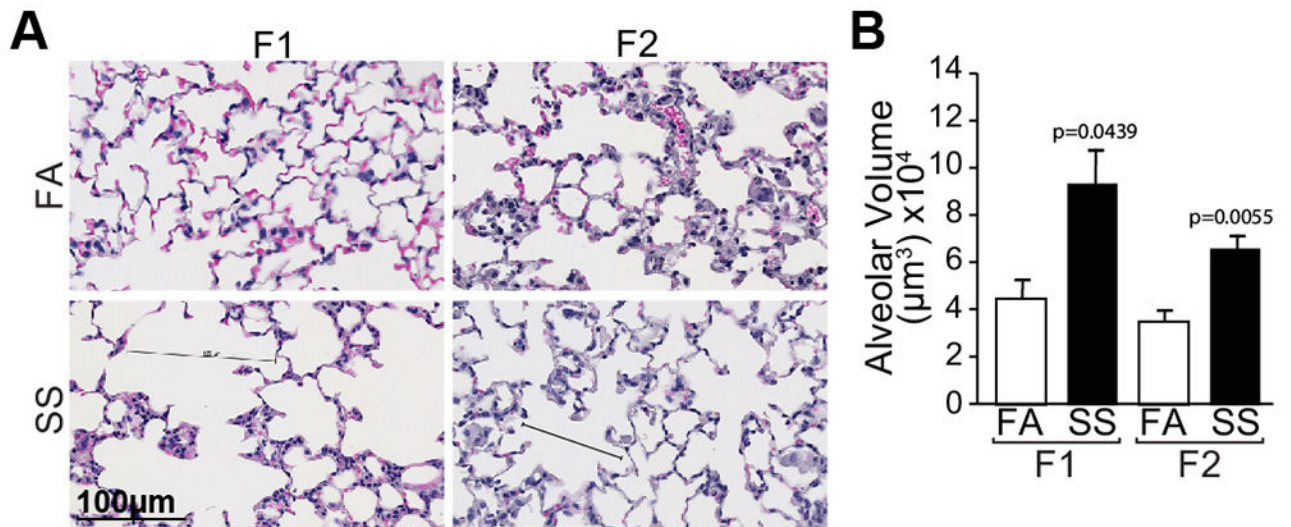
Lung resistance ( $R_L$ ) was measured using FlexiVent system in response to: (A), increasing doses of computer-controlled nebulized methacholine (MCh), (B), dynamic lung compliance ( $C_{dyn}$ ), which reflects lung compliance at any given time during actual movement of air. Determination of  $R_L$  and  $C_{dyn}$  was carried after Af-sensitization as described in Methods. The results are representative of two different sets of inhalation exposures. Data are expressed as mean  $\pm$  SD ( $n = 6 - 17/\text{group}$ ). \*  $p = 0.05$ ; FA = filtered air, SS = sidestream cigarette smoke; F1: first generation; F2 second generation.





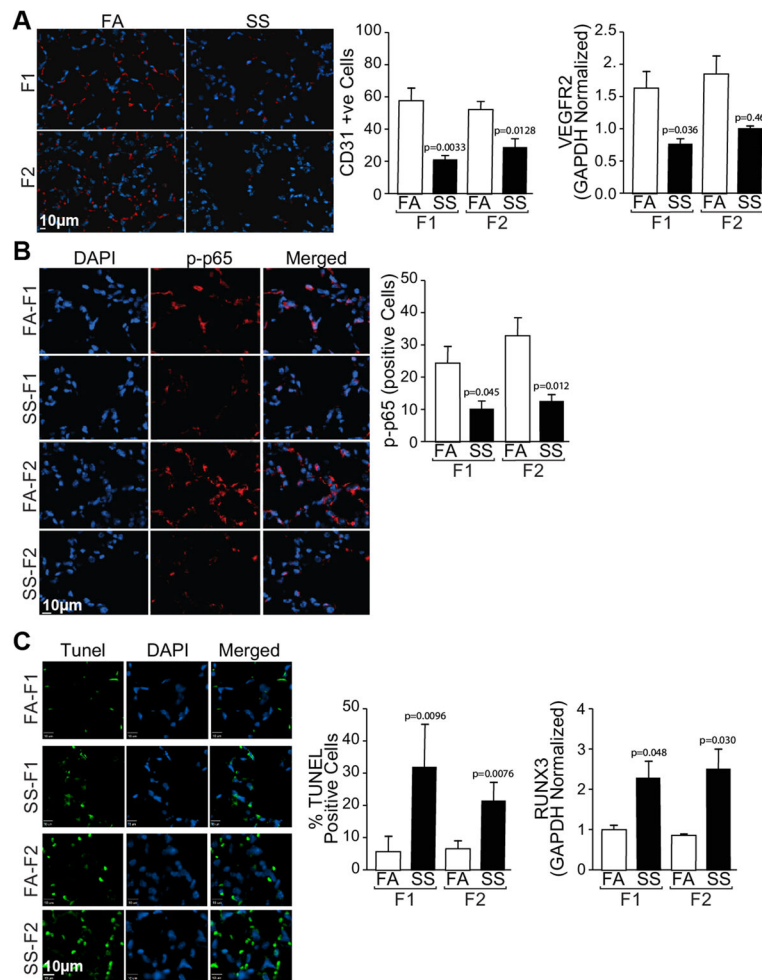
**Fig. 2. Transgenerational effect of gestational SS-exposure on Th1/Th2 cytokines/transcription factors**

**A:** BALF levels of IL-13 (left panel) and IFN- $\gamma$  (right panel) were determined by the Multiplex ELISA kit. **B:** Lung levels of GATA 3 (left panel) and T-bet (right panel) were determined by quantitative RT-PCR analysis total lung RNA from mice sensitized with Af as described in the Methods section. Results are representative of animal responses from two different sets of inhalation exposures. Data are expressed as mean  $\pm$  SD (n = 5–7/group. FA = filtered air, SS = secondhand cigarette smoke; F1: first generation; F2 second generation.



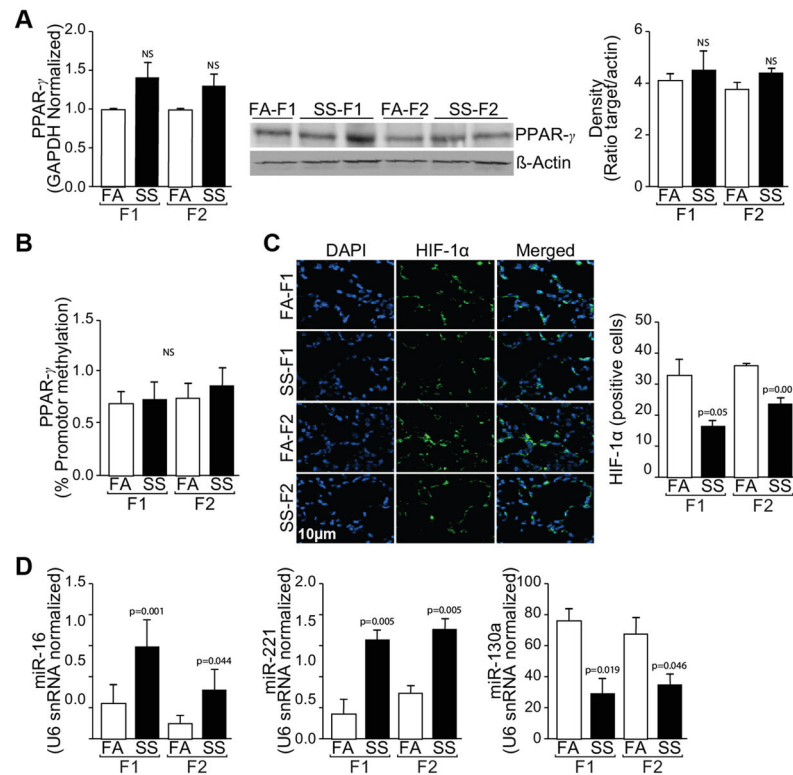
**Fig. 3. Gestational SS-induced alveolar simplification is exhibited by F1 and F2 lungs**

**A:** H&E stained lung sections (5  $\mu\text{m}$ ) from F1 and F2 mice; **B:** quantitation of alveolar volumes. The results are representative of animal responses from two different sets of inhalation exposures. Data are expressed as mean  $\pm$  SD (n =6 –17/group. FA = filtered air, SS = secondhand cigarette smoke; F1: first generation; F2 second generation.



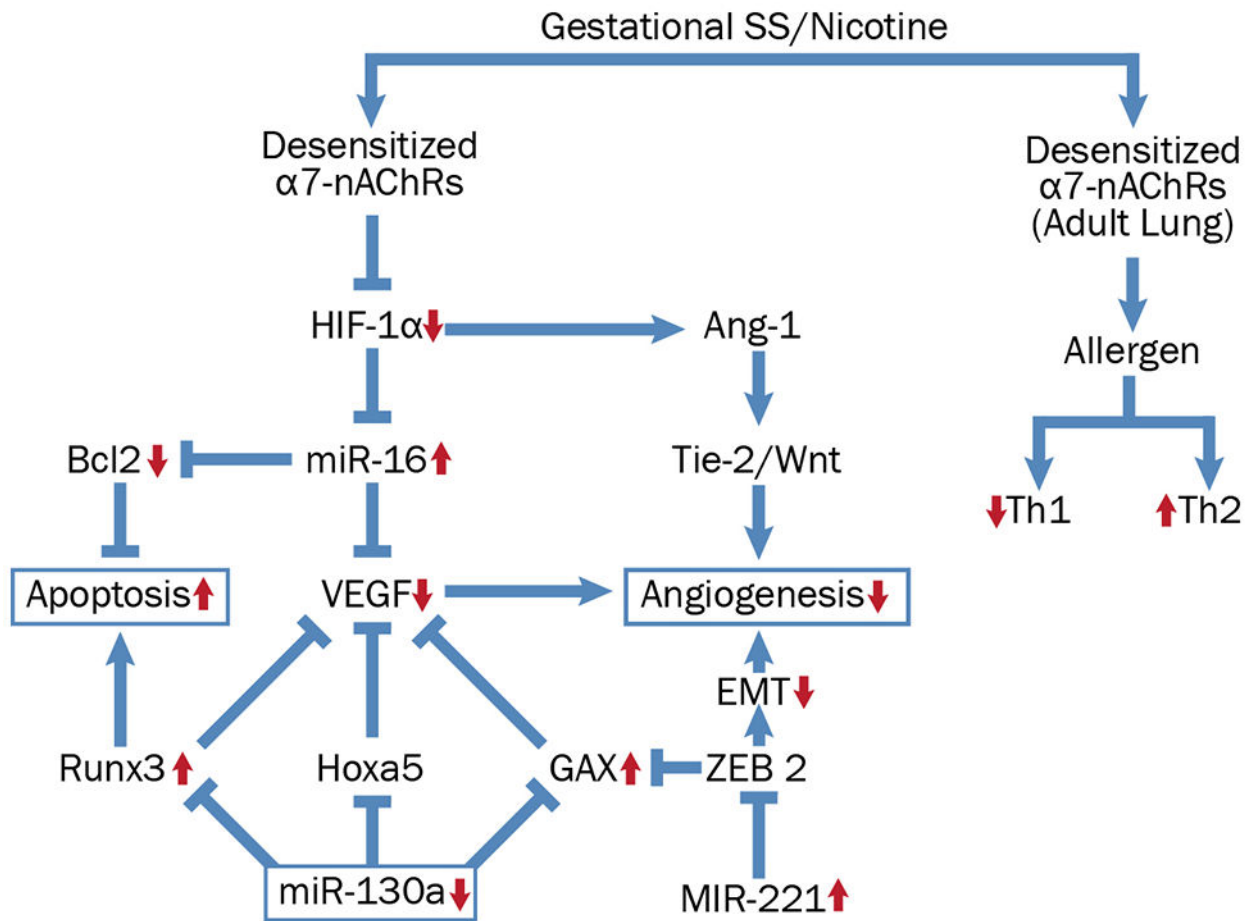
**Fig. 4. Gestational SS-exposure downregulates angiogenesis, VEGFR2, p65-NF $\kappa$ B, and upregulates apoptosis in F1 and F2 lungs**

**A:** Lung sections were stained with anti-CD3 antibody (left panel) and the red fluorescent cells were quantitated microscopically (middle panel); expression of VEGFR2 was done by qPCR analysis (right panel). **B:** Lung sections were stained with phosphorylated(p)-anti-p-65 antibody (left panel) and p-65-positive cells were quantitated (right panel). **C:** Lung sections were stained for TUNEL-positive cells using In Situ cell Death Detection kit (left panel) and quantitated microscopically (middle panel); RUNX3 levels were determined by quantitative RT-PCR using total lung RNA. Where indicated slides were counterstained with DAPI to visualize nuclei. Results are representative of animal responses from two different sets of inhalation exposures. Data are expressed as mean  $\pm$  SD (n = 5–7/group. FA = filtered air, SS = secondhand cigarette smoke; F1: first generation; F2 second generation).



**Fig. 5. Effects of gestational SS on PPAR $\gamma$ , HIF-1 $\alpha$  and miRs in F1 and F2 lungs**

**A:** Quantitation of PPAR $\gamma$  by quantitative RT-PCR of total lung RNA (left panel); Western blot analysis of lung homogenates for PPAR $\gamma$  (middle panel) and ratio of PPAR $\gamma$  : actin from the Western blots by densitometry (right panel). **B:** percent methylation of PPAR $\gamma$  promoter (left panel **C:** Immunofluorescent staining of lung sections for HIF-1 $\alpha$  with DAPI for nuclear staining (left panel) and quantitation of HIF-1 $\alpha$ -positive cells (right panel). **D** Quantitative RT-PCR analysis for miR-16 (left panel), miR-221 (middle panel), and miR-130a (right panel). Data are normalized with snRNA U6. The results are representative of animal responses from two different sets of inhalation exposures. Data are expressed as mean  $\pm$  SD (n = 5–7/group. FA = filtered air, SS = secondhand cigarette smoke; F1: first generation; F2 second generation).



**Fig. 6. A tentative schematic diagram showing possible pathways eliciting the effect of gestational SS exposure**

Desensitization of nAChRs during gestational nicotine/SS-exposure affects HIF-1 $\alpha$  levels increasing apoptosis and decreasing angiogenesis in the lung. The expression of pro-apoptotic miRs (miR-16 and miR-221) is significantly elevated in gestationally SS-exposed. Gestational SS-exposure decreases miR-130a that targets the anti-angiogenic factor GAX and the proapoptotic factor RUNX3 (both GAX and RUNX3 are increased in F1 and F2 lungs). Red arrows indicate established responses with upward arrows indicating significant increase and downward arrows indicating significant decrease in the indicated response. Components in the pathway that are without the arrows predict the indicated response but remain hitherto unproven.